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14. ABSTRACT

We are testing, in prospective studies from Hopkins (Brady) and Harvard (PHS, HPFS), whether the combination of telomere length variability in prostate cancer cells and short telomere length in cancer-associated stromal cells is an independent prognostic indicator of poor prostate cancer outcome. In Year 3, we continued our progress towards a fully optimized protocol for automated slide scanning and multi-channel acquisition of fluorescent images using the TissueFAXS Plus microscopy workstation and TissueFAXS 4.0 software (Tissue Gnostics). We are now able to reliably scan and analyze an entire prostate TMA. We demonstrated our ability to quantitate the telomere signals on a per nucleus basis in both the cancer and cancer-associated stromal (CAS) cells. We developed a set of criteria we will use to decide whether the currently established automated method is sufficiently optimized to move forward with Tasks 5, 6, and 7 in Year 4. Given what we have observed and documented during the automation and optimization process in Year 3, we do not expect that we will need to revert to the manual method of telomere length determination to complete the remaining tasks.

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INTRODUCTION: Currently used clinico-pathologic prognostic factors are imperfect predictors of outcome in the men with clinically localized prostate cancer, the majority of men diagnosed today. Thus, tissue-based biomarkers that significantly enhance the predictive power are urgently needed to improve treatment and surveillance decision-making for these men. To address this pressing clinical need, we have assembled a multidisciplinary prostate cancer research team from Johns Hopkins and Harvard to validate and optimize a novel tissue biomarker of prognosis for men with clinically localized prostate cancer that we recently identified – telomere length variability in prostate cancer cells combined with short telomere length in cancer-associated stromal cells. In our prior work, men with this combination had a substantially higher risk of dying of their prostate cancer compared with men without this combination. Equally importantly, men without this combination rarely died of their prostate cancer over 10 years. Key next steps to realize the great translational potential of telomere length as an independent prognostic tissue biomarker are optimized biomarker assessment and validation. Thus, our aims are to: 1) Optimize the method for assessing telomere length by FISH using a high-throughput approach to yield a test feasible for the clinical setting. 2) Validate our compelling findings in two other cohort studies on prostate cancer outcomes: a) men surgically treated and followed for lethal prostate cancer; and b) men surgically treated and followed for prostate cancer recurrence. 3) Determine optimal biomarker cutpoints for prognosis.

BODY: This work is being performed collaboratively by two institutions: Johns Hopkins Bloomberg School of Public Health and Harvard School of Public Health. This progress report covers Year 3 ("Progress in Year 3"). For completeness and context, we also provide the progress we reported for Years 1 and 2 ("Progress in Years 1 and 2").

Progress in Years 1 and 2:
In Year 1, we obtained all required IRB approvals for both the Brady prostate cancer recurrence Study (Hopkins), the Physician's Health Study (PHS) and the Health Professionals Follow-up Study (HPFS, Harvard), including from the DOD IRB (**Task 1 completed**). Drs. Platz and De Marzo previously created the Brady prostate cancer recurrence nested case-control study (Brady study; in part with prior DOD funding to Dr. Platz at Hopkins) and associated tissue microarrays (TMAs). This TMA set is now part of the Prostate Cancer Biorepository Network (PCBN). For equitable use and tracking purposes, during Year 1, we applied for access to these TMAs and received approval from the PCBN. For the Brady study, we pulled the recurrence TMAs (N=16 TMAs, which includes 524 cases and 524 controls) and cut and mounted the sections (**Task 4b completed**) for staining for telomere-specific FISH, cytokeratin 903 immunofluorescence, and for DAPI.

The PHS and HPFS are existing cohort studies, and TMAs have been constructed for those participants who underwent a radical prostatectomy with other funds, including previous DOD funding (to Dr. Mucci at Harvard). In Year 2, we received sections from the 6 PHS TMAs and 3 HPFS TMAs that had not yet been constructed when we conducted our prior work on the telomere biomarker and that served as the preliminary data for the proposal for the current study (**Task 4a completed**).

In Year 1, the Johns Hopkins investigators purchased a new state-of-the art fluorescence slide scanner and associated image analysis software from Tissue Gnostics using donor funds (**Task 2a completed**). This new system uses the TissueFAXS Plus (Tissue Gnostics, Vienna, Austria) microscopy workstation for slide-based cytometry of tissue sections and TMAs. The microscope is a Zeiss Z2 Axioimager with high quality optics applicable for fluorescence imaging. The microscope is fitted with the following filter sets: DAPI, Alexa 488/Cy2, Alexa 568/Cy3, and Alexa 633/Cy5. The system contains an ultra-precise motorized stage for 8 slides for high throughput scanning. In addition, a separate image analysis workstation contains a high-performance computer workstation (HP Z420 configured with 6 cores) and includes the TissueFAXS 4.0, TissueQuest 4.0, and HistoQuest 4.0 software modules.

In Year 2, we developed and began to optimize protocols for fully automated slide scanning and multi-channel acquisition of fluorescent images using a 40X oil objective. We demonstrated the ability to image prostate tissues that have been fluorescently co-stained with a Cy3-labelled telomere specific peptide nucleic acid probe (red), a prostate basal cell-specific anti-cytokeratin primary antibody and detected with a fluorescent secondary antibody conjugated to Alexa Fluor 488 (green), and counterstained with DAPI to detect the nuclei (blue). In addition, we utilized the Tissue Gnostics image analysis software to begin to automate the segmentation of individual cell nuclei and telomere FISH signals (**Task 2b completed**).

Progress in Year 3:

In Year 3, we further refined and are nearing completion of fully optimized and automated imaging parameters, followed by single cell telomere length analyses. First, we continued our progress towards a fully optimized protocol for automated slide scanning and multi-channel acquisition of fluorescent images using the TissueFAXS Plus microscopy workstation and TissueFAXS 4.0 software (Tissue Gnostics). We are now able to reliably scan and analyze an entire prostate TMA. First, as shown in Figure 1, we acquire a 10X magnification pre-scan image using the DAPI channel.

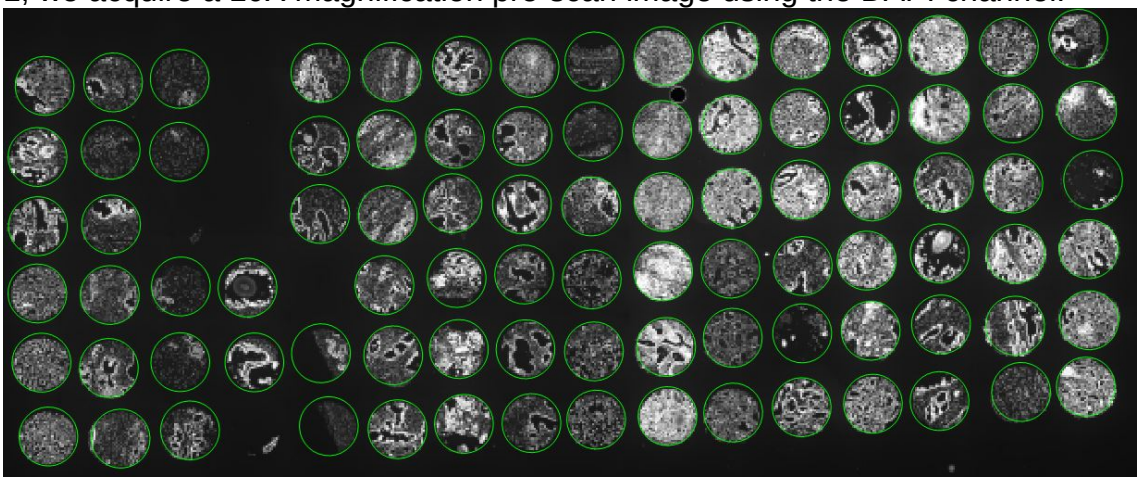

Figure 1. Selection of TMA spots of interest from HPFS test TMA. This representative image shows the TMA spots identified on a 10X magnification pre-scan (DAPI channel).

Next, we image the entire TMA at 40X magnification (sufficient for image analysis) by collecting images on 4 different channels (DAPI, Cy2, Cy3, Cy5), followed by segmentation of the images into specific TMA spots.

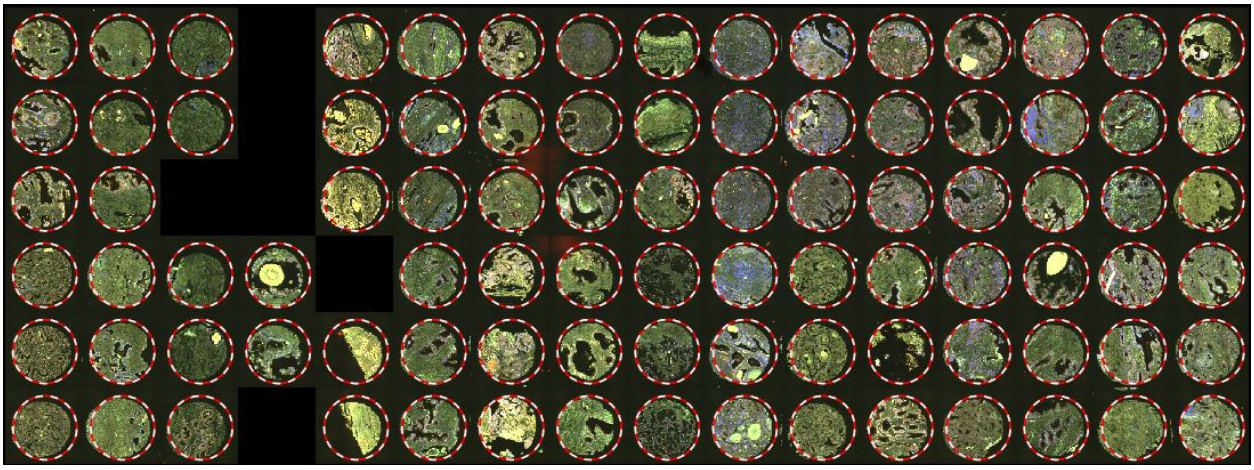

Figure 2. Imaging of TMA spots and segmentation into specific TMA spots. This representative image shows a TMA, with the TMA spots imaged and segmented at 40X magnification.

Next, zooming into a specific TMA spot (top row; 12th column), we are able to demonstrate the detection of individual cells of specific cell types, and perform image analysis for these specific cells types. This step was significantly enhanced by adding the prostate epithelial-specific marker NKX3.1 (detected in the Cy5 channel) to our antibody panel in the multiplex staining.

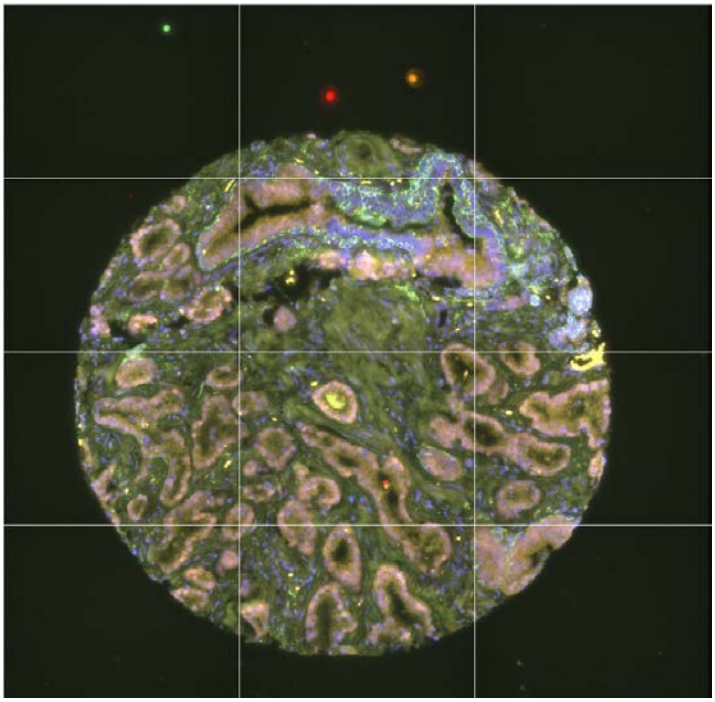

Figure 3. Telomere-specific FISH and immunostaining in a prostate TMA spot. This representative image contains both normal prostate glands (basal and luminal cells) embedded in prostatic stroma and an area of tumor cells/glands in areas of cancer-associated stroma. DAPI (blue), telomeric DNA (red), and basal cells are demarcated with a basal cell-specific cytokeratin antibody (green). NKX3.1 immunostaining (orange) highlights cancer cells and normal luminal cells. Note: at this power, the telomeric DNA (red) is not visible. The overall image is stitched together and each panel outlined (12 in all) represents an image at 40X magnification.

Next, by zooming into the above image and shown in Figure 4, we are able to demonstrate the ability to visualize the overlay image (upper left panel) that consists of the individual channels; DAPI (nuclei; upper right panel), Cy5 (NKX3.1; lower left panel), and Cy3 (telomeres; lower right channel).

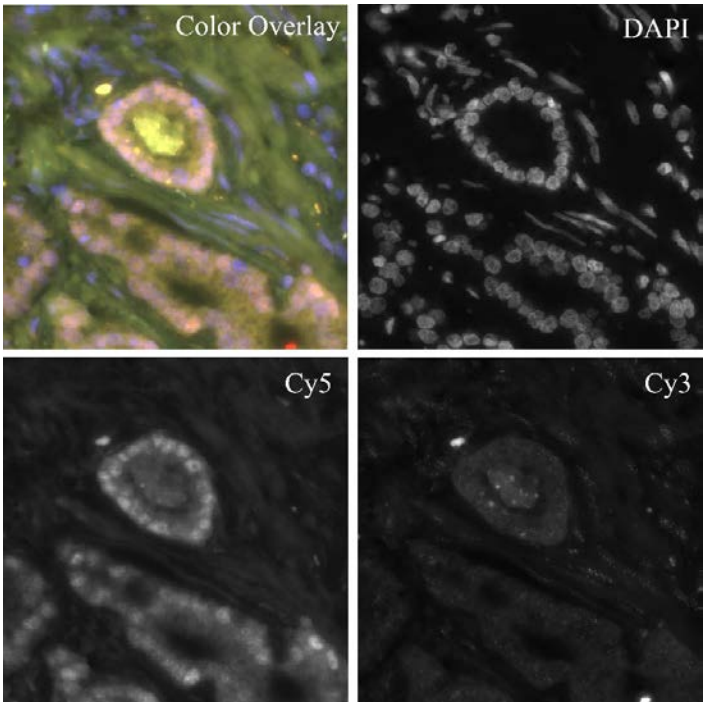

Figure 4. Representative region containing cancer cells and cancer-associated stromal cells. These representative images contain both cancer cells (Cy5-positive) and cancer-associates stromal cells (Cy5-negative). The overlay image is presented, along with monochrome images of the DAPI (nuclei), Cy5 (NKX3.1 protein), and Cy3 (telomeres) channels.

From these collected images, as shown in Figure 5, we have developed (i) nuclear segmentation parameters based on the DAPI and Cy5 channels that specifically identify cancer cell nuclei (red circles; left panel) and (ii) a specialized algorithm to detect the telomeres within the nuclei (yellow dots; right panel).

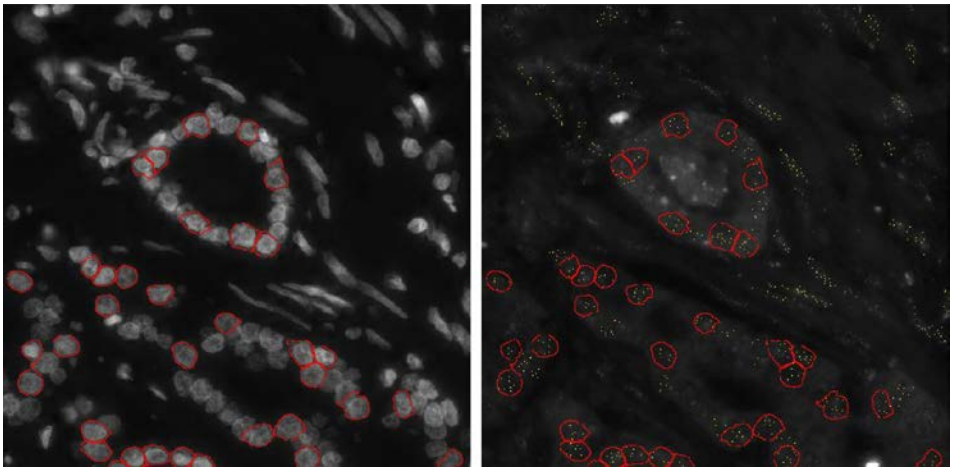

Figure 5. Example of automated cancer cell segmentation and telomere detection. An example of the nuclear segmentation parameters based on the DAPI and Cy5 channels that specifically identify cancer cell nuclei (red circles; left panel) and a specialized algorithm to detect the telomeres within the nuclei (yellow dots; right panel) are shown.

In addition, as shown in Figure 6, we have developed nuclear segmentation parameters based on the DAPI channel and lack of signal in the Cy5 channel that specifically identifies cancer-associated stromal nuclei (red circles; left panel) and the specialized algorithm detects the telomeres within the nuclei (yellow dots; right panel).

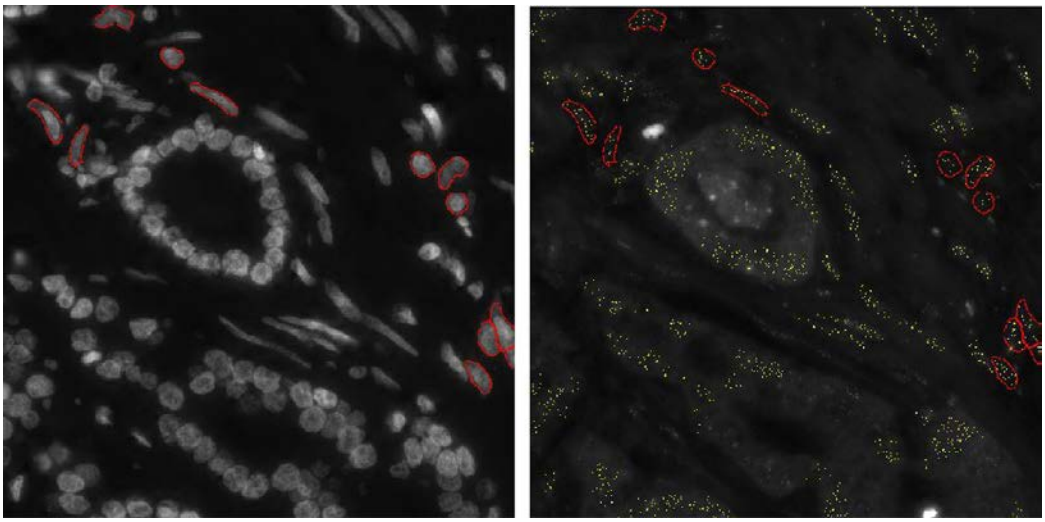

Figure 6. Example of automated cancer-associated stromal cell segmentation and telomere detection. An example of the nuclear segmentation parameters based on the DAPI and Cy5 channels that specifically identifies cancer-associated stromal cell nuclei (red circles; left panel) and a specialized algorithm to detect the telomeres within the nuclei (yellow dots; right panel) are shown.

Finally, we are able to demonstrate the ability to quantitate the telomere signals on a per nucleus basis in both the cancer and cancer-associated stromal (CAS) cells. Telomere-specific FISH signals are linearly proportional to telomere length and thus, telomere length can be quantified via digital image analysis. Telomere length, on a per cell basis, is determined as the ratio of the total intensity of telomeric signals in each cell to the total intensity of the DAPI stained nuclear DNA signal in the same cell. As an example (Figure 7), we quantitated the telomere lengths in the same TMA spot depicted in Figure 3 and as expected, signals were less intense (i.e., telomere lengths were shorter) in cancer cells when compared to the mean telomere ratio in the CAS cells (5.72 vs. 17.53, respectively).

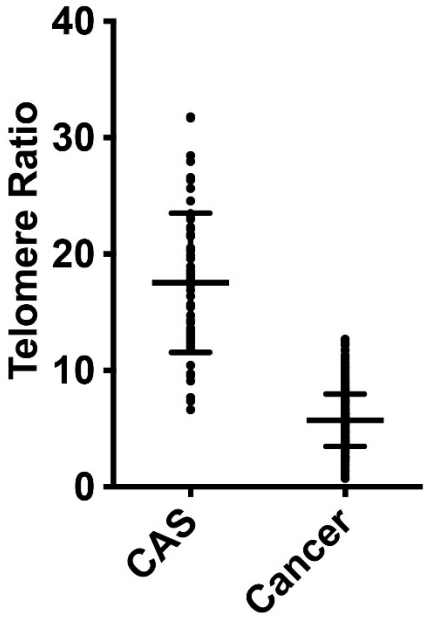

Figure 7. Distribution of telomere length ratios from one TMA spot for cancer-associated stroma (CAS) and cancer cells. For each selected cell, the telomere ratio was determined by summing the individual telomere intensities ("telomere sum"), and this total was divided by the total DAPI fluorescence signal ("DAPI sum") for that same nucleus. The mean and standard deviations are depicted for each cell type.

Next steps and plans for Year 4:
The Hopkins and Harvard investigators met in person at the annual Prostate Cancer Foundation Retreat (no cost to DOD for travel) to review and summarize Year 3 progress for this progress report. At this meeting, we determined that Task 3 (*Test the automated method of telomere length determination for precision and validity by rerunning the automated method (precision) and by comparing to the nonautomated method (validity)*), which would have involved rescanning the original 5 HPFS TMA sections to compare the automated method to the nonautomated method of telomere length determination for precision and validity, would not be informative. During optimization of the method, we concluded that the process of capturing the TMA images adversely affects the fluorescence of both the Cy3 telomere signals, as well as the DAPI DNA signals, in nonsystematic ways, due to photobleaching. We will instead perform Task 3 using 2 (of 7) adjacent sections of the test TMA provided by Harvard (contains HPFS prostate cancer and normal adjacent tissue).

During our meeting, we also decided that at the beginning of Year 4, we will request the PHS TMAs and the Brady TMAs be cut and given to us (**Task 4**). We have already requested and received the 2 additional new HPFS TMAs. Furthermore, we developed a set of criteria we will use to decide whether the currently established automated method is sufficiently optimized to move forward with Tasks 5, 6, and 7 in Year 4. Using a test TMA provided by Harvard (constructed using the same prostate cancer tissue repository as in the HPFS), we will assess the following: 1) Whether FISH/ immunofluorescence appropriately labels the telomeres, centromeres, and specific cell types (basal epithelial, luminal epithelial, cancer), 2) Whether 7 in-focus images for each channel (DAPI, Cy2, Cy3, Cy5) are captured without signal intensity saturation and/or overexposure, 3) Whether the individual TMA spots can be clearly segmented (demonstrated in Figure 2), 4) Whether the labeling and quality of the image capture is sufficient for the Tissue Gnostics software platform to perform image analysis of telomere signal intensity for individual cells and types of cells, 5) Whether the software parameters (e.g. nuclei size, nuclei compactness, sensitivity threshold of dot detection, etc.) are optimized for the different signal intensities of cancer cells (dim) versus cancer-associated stromal cells (strong), and 6) Whether all of the steps above produce a reproducible telomere length values. With respect to 6), we will determine the reproducibility of the staining, image capture, and image analysis of two adjacent sections performed on 2 different occasions. If after reviewing the results of the test TMA sections, our team members are in agreement that the methods are sufficiently valid, robust and reproducible, we will then proceed with the optimized, automated method of telomere length determination on the cohort TMAs. Given what we have observed and documented during the automation and optimization process in Year 3, we do not expect that we will need to revert to the manual method of telomere length determination to complete the remaining tasks.

KEY RESEARCH ACCOMPLISHMENTS: None directly from this project to date (see above).

While we are pursuing the aims of the current DOD grant, our telomeres and prostate cancer team continues to conduct research addressing the aims and related questions using the data collected under our two prior DOD grants on telomeres (W81XWH-06-1-0052, W81XWH-05-1-0030).

Joshu CE, Peskoe SB, Heaphy CM, Kenfield SA, Van Blarigan EL, Mucci LA, Giovannucci EL, Stampfer MJ, Yoon G, Lee TK, Hicks JL, De Marzo AM, Meeker AK, Platz EA. Prediagnostic Obesity and Physical Inactivity Are Associated with Shorter Telomere Length in Prostate Stromal Cells. Cancer Prev Res (Phila). 2015 Aug;8(8):737-42. doi: 10.1158/1940-6207.CAPR-15-0097. Epub 2015 May 19. PubMed PMID: 25990087; PubMed Central PMCID: PMC4526348.

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Heaphy CM, Zarinshenas R, Baena-Del Valle JA, Kulac I, Graham MK, Joshu CE, De Marzo AM, Platz EA, Meeker AK. Tissue-based telomere length measurements as a biomarker for individualized prostate cancer risk stratification and prognostication (poster). Annual Prostate Cancer Foundation Scientific Retreat. October 8-10, 2015, Washington, DC.

REPORTABLE OUTCOMES: None

CONCLUSIONS: None to date, as consistent with the Statement of Work.

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APPENDICES: None

SUPPORTING DATA: None